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Contribution of the Leukocyte Adherence Inhibition Test to the Diagnosis of Non—IgE-Mediated Immunoreactivity against Cochineal Dye

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) for evaluating immunoreactivity against cochineal dye (E120) in patients with clinical suspicion of symptomatic hypersensitivity to this food additive.

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Study Design: We retrospectively examined the medical charts of a population of 82 patients diagnosed with Atopic Dermatitis and/or Urticaria with clinical suspicion of cochineal hypersensitivity investigated with an *ex vivo* challenge monitored by LAIT against a commercial preparation of cochineal dye provided for butcheries.
Place and Duration of Study: Instituto Alergoimuno de Americana – São Paulo – Brazil – between January 2018 and July 2023.
Methodology: The percentage of Leukocyte Adherence Inhibition (LAI) promoted by the *ex vivo* challenges with cochineal dye was distributed in ranges through a cascade distribution chart to outline the variability of the results.
Results: The LAI mean was 42.6%; SD 28.8%, ranging from 0% to 98%; mode = 0% (appeared 15 times). There was a wide range of distribution of LAI results, suggesting that some patients had immunoreactivity against the cochineal allergens while others did not.
Conclusion: Our preliminary results support that the LAIT performed with cochineal dye may differentiate diverse degrees of *ex vivo* immunoreactivity against this food colorant in allergic patients.

Keywords: Allergy; atopic dermatitis; carminic acid; cochineal dye; dactylopius coccus; diagnosis; food additives; hypersensitivity; leukocyte adherence inhibition test; metabolic syndrome; non—ige-mediated immunoreactivity; urticaria.

1. INTRODUCTION

Food additives are non-nutritional ingredients intentionally added to foods (and drinks) to modify their physical, chemical, biological, and/or sensory characteristics. Several color additives broadly used in industrialized foods are associated with allergic reactions in unsuspecting consumers [1]. World-famous alcoholic and non-alcoholic beverages include in their ingredients allergenic color additives, such as cochineal dyes, in their composition [2,3]. Cochineal dyes and pigments have been producing allergies through industrialized foods (yogurts, refreshment drinks, candies, sauces, sausages, processed meats, kebabs, processed fruits, liqueurs, bitters), in cosmetics (nail polish, lipstick, hair tinctures), personal hygiene products (toothpaste, soaps, shampoos), paints (clothing fabric, watercolor, oil paints), quasi-drugs (dental plaque dyeing agents), and pharmaceutical preparations, penalizing sick people trying to treat their diseases with colored medicines [4-6]. Cochineal dye allergy is also an occupational hazard, mainly for workers from the dye industry, textile industry, food handlers, and butchers who use it to color sausages [7-10].

Cochineal insects (*Dactylopius spp.*) are scale insects (mealybugs) of the order *Hemiptera*, suborder *Sternorrhyncha* that parasitizes cacti [11]. The "true cochineal" (*Dactylopius coccus var.* Costa), also known as "American cochineal," is a parasite of the prickly pear cactus that produces excellent amounts of carminic acid, a red dye commercially extracted for several purposes [12]. The Aztec, Mayan, and Inca peoples first used cochineal dyes. They were later commercially exploited by Spanish colonizers as "America's red gold," a high-value trade good exported to Europe [13,14]. Cochineal extract is the raw material extracted from the dried bodies of insects. In contrast, carminic acid (the main active color ingredient of cochineal extract) is an anthraquinone-derivative (9,10-anthraquinone-2-carboxylic acid linked to a methyl group, a glucopyranose, and four hydroxyls). Carminic acid is a natural defense weapon pregnant cochineals produce as a chemical deterrent against ant predation [15]. With a molecular weight of 492.4 Da, carminic acid stays in equilibrium with two isomers and is the most well-studied anthraquinone of animal origin [16, 17]. Dye producers also extract carminic acid and similar anthraquinone-derivatives from other scale insects such as *Kermes vermilio* (kermes), *Porphyrophora polonica* (Polish cochineal), *Porphyrophora hamelii* (Armenian cochineal), and *Kerria lacca* (lac) [18]. The "red insect dyes" anthraquinones comprise the cochineal dye (carminic acid), the kermes dye (kermesic acid), and the lac dye (laccaic acid) [19]. Cochineal extract is originally prepared through aqueous-alcohol solutions, usually consisting of 10% carminic acid plus the residual insect bodies. Nowadays, several public and trade secret techniques are industrially employed to produce diverse preparations at a commercial scale [20].

The designation "carmine" is academically applied to a calcium salt of an aluminum complex

of carminic acid [21]. When precipitated with calcium and aluminum (or other metal ions), carminic acid (a soluble dye) turns into a dimeric or tetrameric insoluble pigment (carmine) [22]. By definition, a dye is a water-soluble colorant that usually bonds chemically to the substrate. At the same time, pigments are insoluble-in-water particulate colorants that need the addition of a binder to fix the material they color [23]. Lake pigments are dyes prepared by adsorbing or precipitating an organic dye onto an inorganic substrate; the carminic aluminum lake is formed by the aluminum atom chelated with two molecules of carminic acid [24]. Besides the raw cochineal extract, the carminic aluminum lake is also a food colorant [25].

Carmine and, by extension (not so academically) carminic acid are cataloged by the Colour Index (CI) as "Natural Red n° 4" (CI 75470) [26]. The Colour Index denomination is shared by the paint industry, the textile industry, the cosmetic industry, and, sometimes, by the tattoo business that also employs allergenic cochineal dyes in its practice [27]. However, carminic acid changes its fugitive color according to the pH. When dissolved in acid solutions, carminic acid is orange; at neutral pH, it turns red; and, in alkaline solutions, it turns violet [18]. When heated in the presence of ammonia, carminic acid forms amino carminic acid, a not consensually approved derivative that keeps the red color at a very low pH, which is used (sometimes illicitly) in some countries as an "acid-stable carminic acid" to color acidic food [28, 29]. The Codex Alimentarius of the World Health Organization lists the food and pharmaceutical additives derived from cochineal extracts (not so academically) as "carmines" under code E 120 [30].

Anthraquinone derivatives are causes of allergic and phototoxic contact dermatitis [31, 32]. To study the immunoreactivity of haptens, one must employ techniques to conjugate them with human proteins [33]. Swiss and German researchers first investigated patients with clinical symptoms of allergy elicited by cochineal extracts with solid-phase radioimmunoassay for specific IgE with disks incubated with cochineal extract and disks incubated with carminic acid conjugated to Human Serum Albumin. Some patients reacted against the cochineal extract, while others responded to the hapten-carrier conjugate [34]. Cochineal dyes may produce allergic reactions elicited by hypersensitivity against insect proteins, hypersensitivity against insect proteins complexed with carminic acid, and hypersensitivity against human proteins complexed with carminic acid [35]. An ultrafiltration technique, followed by a silver-stained SDS-PAGE, was designed to identify the insect proteins in the cochineal extract [36]. Turkish researchers also attributed allergic reactions to cochineal extracts to insect protein residues found in the cochineal extracts [37]. Other allergic reactions were attributed (not so convincingly) to carminic acid [38]. Theoretically, the low molecular weight (492 Da) carminic acid is not allergenic and would act as a hapten in allergic individuals to produce hypersensitivity reactions [39]. Haptens are low-molecular-weight molecules able to be covalently bound to a heavier carrier [40]. Japanese researchers have demonstrated that, in solution, carminic acid presents itself as multivalent aggregates that readily bind to proteins such as albumins and globulins to act as haptens in allergic patients [41]. At physiologic conditions, molecules over 150 Da do not permeate through enterocytes into the bloodstream [42]. However, under inflammatory conditions, a hyperpermeability state may allow the permeation of heavier molecules between damaged enterocytes [43]. Once free in the bloodstream, with four free hydroxyls, a carboxylic acid, and a digestion-resistant glucopyranose, carminic acid is eligible to link to human serum proteins, deforming its tertiary structure to establish hapten-carrier complexes able to elicit (IgE-mediated or non—IgE-mediated) antibodies responses. One can also realize that when conjugated with a heavy metal such as aluminum, the chance of this happening is more remarkable, as has already been demonstrated for nickel and cobalt [44, 45].

Japanese researchers demonstrated, by immunoblotting, that proteins contaminating the cochineal dyes caused the clinical symptoms of three patients presenting urticaria after the ingestion of drinks with cochineal dyes, which were IgE-sensitized to the same 38 kDa protein (CC38K, probably a phospholipase) marked in the SDS-PAGE [46]. Spanish researchers reported ten workers from a factory of cochineal dyes who had work-related symptoms of rhinitis and asthma. Specific IgE against a protein (with molecular weight between 10 and 30 kDa) was found only in one worker. They also found (by ELISA) the presence of specific IgG against the cochineal extract in all ten subjects [47]. This same group reported three other patients with work-related symptoms of rhinitis and asthma associated with inhalation of cochineal dyes. In

the raw cochineal extract, they identified, by SDS-PAGE, three protein bands of 17 kDa, 28 kDa, and 30 kDa. In the boiled cochineal extract, they identified a band of 50 kDa. In the commercial "water-soluble carmine" used by the workers, they identified two protein bands of 28 kDa and 50 kDa. Immunoblotting detected specific IgE binding at the 17 kDa band in cochineal raw extract, the 50 kDa band in the boiled one, and the 28 kDa band in the "water-soluble carmine" [48].

Despite the hypersensitivity question, as an insect-derived food additive, vegetarian and vegan consumers do not accept cochineal derivatives. They must not be present in Kosher and Halal foods. Several laboratories developed methods to specifically detect carminic acid in food, including for religious food certification [49-54]. To avoid the inconvenience of insect protein contamination and satisfy diets restricted by personal or religious convictions, microbial factories are developing the heterologous reconstruction of the carminic acid biosynthetic pathway for the *de novo* biosynthesis of carminic acid [55-59]. Patients with an allergy to House Dust Mites commonly also have an allergy to the insect proteins in the cochineal dyes. However, hypersensitivity to cochineal dyes does not depend on concurrent mite allergy [60]. The cross-reacting agent is tropomyosin, an evolutionary myofibrillar protein similar in insects, mites, and crustaceous [61].

To evaluate the potential of the Leukocyte Adherence Inhibition Test (LAIT) as a tool for assessing immunoreactivity against the cochineal dye, we retrospectively examined the medical charts of patients with diagnosis of urticaria and/or atopic dermatitis with clinical suspicion of allergy to red food additives (whose skin tests were not reactive and the specific IgE undetectable for cochineal dye) who were investigated with an *ex vivo* challenge monitored by LAIT against a commercial aqueous preparation of cochineal extract provided for sausage production.

2. MATERIALS AND METHODS

2.1 Subjects

After receiving Institutional Review Board approval from the Instituto Alergoimuno de Americana (Brazil; 04/2023), we proceeded with the electronic chart review of a population of 7,400 allergic patients who attended our outpatient facility from January 2018 to August 2023. We identified a cohort group of 82 patients submitted to an *ex vivo* allergen challenge test with cochineal extract monitored with LAIT. It was a very diversified cohort with 28 males; mean age 37 years; SD 23 years; range 1 to 79 years; modes = 23 and 36 years (each appeared four times); geometric mean = 23.4 years. We offer this procedure o patients with atopic dermatitis and/or recurrent urticaria strongly associated with the ingestion of red-colored industrialized foods, and an inconclusive investigation performed with allergic skin tests and the negative research of specific IgE against cochineal dye performed by ImmunoCAP® [62].

2.2 Antigen Preparation

We acquired the cochineal "natural" extract (3%) from a local butcher supplier (SBR Foods – Conatril – Rio Claro), provided with the proposal of coloring meat, sausages, and dairy products. The extract (100µL) was diluted into 100 mL of a buffer solution [NaCl 10g; KH_2PO_4 0,72g; Na_3PO_4 2,86g; H_2O 600mL] and employed in the LAIT and the research of precipitins.

2.3 Ex vivo Investigation: Leukocyte Adherence Inhibition Test

We performed the LAIT as previously described [63-72]. Shortly, each donor's fresh plasma was divided into two parts and used in paralleled *ex vivo* challenging tests with cochineal dye and the unchallenged plasma assay. We collected the plasma with high leukocyte content (buffy coat) from the heparinized tube after one hour of sedimentation at 37 °C and aliquots of 100 µL. We distributed it into Eppendorf tubes and kept it under agitation for 30 minutes (200 rpm at 37 °C) with (or without, as used as control) antigen extract (10µL of a solution with 1mg/mL and pH 7.5). After incubation, the plasma was allocated into a standard Neubauer hemocytometer counting chamber with a plain, non-metallic glass surface and left to stand for 2 hours at 37 °C in the humidified atmosphere of the covered water bath to allow leukocytes to adhere to the glass. Next, we counted the leukocytes, removed the coverslip, and washed the chamber by immersion in a beaker with PBS at 37 °C. Then we added a drop of PBS to the hemocytometer's chamber and placed a clean coverslip over it. The remaining cells were counted in the same squares as previously examined. The percentage of Leukocyte Adherence (LA) of each assay was estimated as: (the number of leukocytes

observed on the hemocytometry chamber after washing divided by the number of leukocytes observed on the hemocytometry chamber before washing) and multiplied by 100 (%). The Leukocyte Adherence Ratio (LAR) was estimated based on the ratio between the LA from the antigen-specific challenged groups and the LA from the unchallenged control group: LAR = LA of the challenged sample divided by LA of unchallenged control sample; multiplied by 100 (%). To further calculate the Leukocyte Adherence Inhibition (LAI), we subtracted the LAR from 100 (%). Then, we employed the LAI results for the statistics calculations and the cascade distribution chart.

3. RESULTS

As a retrospective survey, there was no research protocol. Therefore, we report the incidental immune investigation as registered in the digital medical charts. The mean LAI was 42.6%; SD 28.8%, ranging from 0% to 98%; mode = 0% (appeared 15 times).

There was a wide range of distribution of LAI results, as outlined by the cascade distribution chart in Fig. 1. Fifteen patients ignored the presence of the allergen on the plasma and presented no inhibition of leukocyte adherence after contact with the cochineal extract (18.3% of the tests). Some patients showed strong immunoreactivity during the *ex vivo* challenge test against cochineal dye. In contrast, others displayed a low or moderate immunoreactivity that possibly would reflect the allergic symptoms after exposure to the allergen.

4. DISCUSSION

Carminic acid is a potential therapeutic strategy for treating Metabolic Syndrome and Non-Alcoholic Fat Liver Disease [73]. Carminic acid also acts as an antioxidant, protecting DNA and erythrocytes against radical-induced oxidation [74]. These potential employs of carminic acid are yet under animal experimentation and need to be evaluated before use in humans, mainly because carminic acid (based on its molecular weight) is not (theoretically) absorbed by the intestinal mucosa under physiologic conditions, and there are no sufficient studies yet about its human pharmacokinetics [75]. However, in the near future, it is possible that from an aesthetic food additive, carminic acid may be upgraded to an essential medicine indicated to treat a condition that affects one-quarter of the human population. The antioxidant capacity of carminic acid is also a chemical stabilizer of pharmaceutical excipients [76]. These are additional reasons for concern about cochineal allergy, a common and overlooked cause of immediate and delayed allergic reactions that must always be suspected in patients with recurrent and chronic allergic symptoms, significantly when the reactions are associated with contact with red pigments or the ingestion of red-colored foods [77].

Sensitization against cochineal components may happen by ingestion, by the skin (through prolonged contact with cosmetics), or by inhalation (occupational hazard) [78]. The IgE-mediated cochineal extract allergy is quickly investigated by cutaneous skin tests at an outpatient Allergology clinic or by automatized ImmunoCAP®, easily offered by most clinical laboratories. However, non—IgE-mediated cochineal hypersensitivities are not consistently recognized by physicians since the laboratory methods designed to diagnose these conditions are not universally available [79]. In the research field, researchers may employ optional elaborated techniques to evaluate hypersensitivities not detectable by the standard clinical laboratories, such as the Basophil Activation Test, the Lymphocyte Stimulation Test, the Leukocyte Migration Inhibition Test, or the LAIT [80-85].

Most of the knowledge about hapten-induced hypersensitivity derives from murine studies [86]. Low-molecular haptens must bind to proteins to interact with immune cells [87]. As a hapten, carminic acid links to host proteins, inducing the production of antibodies developing (IgE and Non-IgE) antibody-mediated hypersensitivities, classified as Types I, II, and III by Gell & Coombs, observable with an optical microscope with the help of the LAIT [88, 89]. The LAIT observes the resultant behavior of leukocytes (adherence inhibition under contact with tested antigen) common to several immune pathways, giving us a clue about the possible culprits [90-93].

Using *ex vivo* challenges to investigate immunoreactivity over haptens brings a technical advantage over the traditional methods since it allows a thirty-minute incubation of the hapten with the patient's plasma. This incubation, theoretically, is sufficient to enable the binding of the haptens to the patient's proteins assembling the hapten-carrier antigen to which the immune

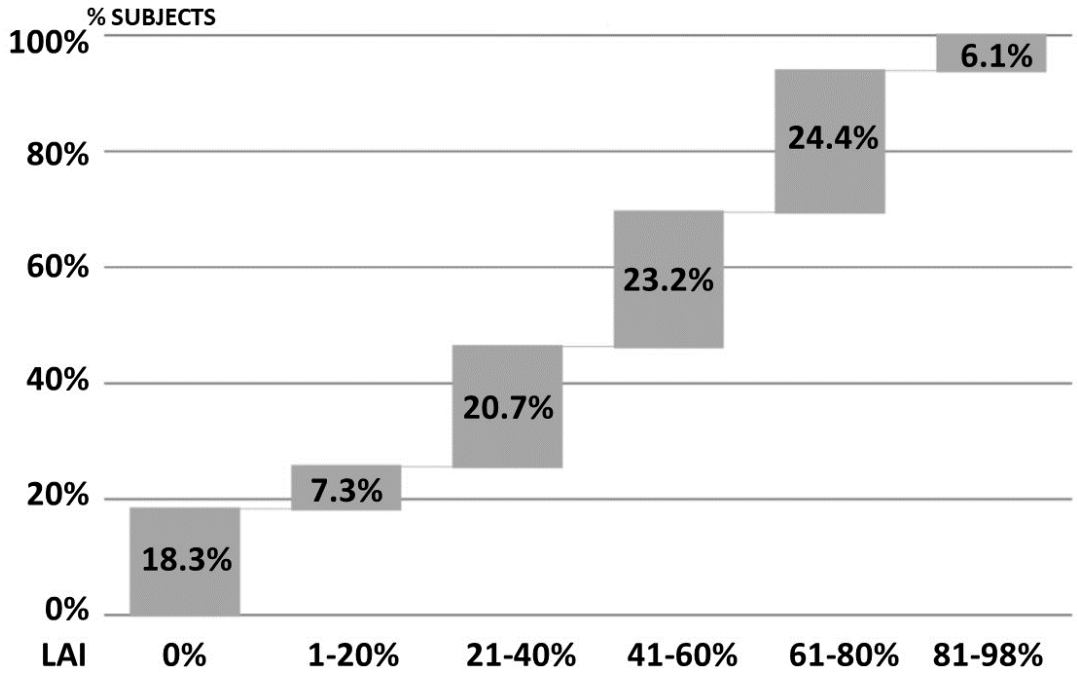

Fig. 1. Cascade distribution chart of the range groups of Leukocyte Adherence Inhibition (LAI) results (x-axis %) of *ex vivo* cochineal dye challenges monitored by the Leukocyte Adherence Inhibition Test (LAIT), according to the respective percentage of results over 82 tests (y-axis)

system is sensitized [94]. If this is the reality, the LAIT is a broader sensitive test than the solid-phase research of serum-specific antibodies, which does not demonstrate immunoreactivity against the hapten-carrier conjugates assembled with the patient's plasma proteins. In our opinion, the demonstration of the assembly of hapten-carrier conjugates with plasma proteins, as well as their *ex vivo* immunoreactivity, should be an obligatory step to evaluate the human pharmacokinetics of any drug before being employed in clinical use.

This preliminary retrospective survey has demonstrated a great range of results against the ex vivo challenge against cochineal extract in a group of allergic patients. It suggests that some patients had previous immunological experience with their proteins or carminic acid. Our Institute has employed LAIT as a complementary triage test to select worthwhile antigens to proceed with the more exhaustive *in vivo* provocations. More studies with prospective larger double-blind cohorts need to evaluate the potential contribution of LAIT in managing patients with cochineal allergy.

5. CONCLUSION

Our preliminary results support that the LAIT performed with cochineal dye may differentiate diverse degrees of *ex vivo* non—IgE-mediated immunoreactivity against the allergens present in the extract and produced in patients' serum. Immunoreactivity against the cochineal extract, as demonstrated by the LAIT, is an indicator of a previous experience of the immune system with this agent. The LAIT positivity does not indicate if there was a skin sensitization, an airway sensitization, or an increase in intestinal permeability, allowing the access of these components into the bloodstream. The LAIT positivity also does not prove that the symptoms that motivate the patient to seek medical help happened due to an immune interaction with the tested antigen. The information given by the LAIT is just that there was a previous immune experience with this allergen which produced an immune memory. The extension of that experience, as suggested by the intensity of the *ex vivo* response, the real-world contact with the agent, the exclusion of the agent from the patient's life, and the close observance of the symptoms after its re-introduction should provide a more confident diagnosis over the participation of the allergen as an eliciting cause of the patient's allergies.

CONSENT

As a retrospective survey of results recorded *incognito*, consent was given collectively by the

institution's ethics committee following the principles of the Declaration of Helsinki [95].

ETHICAL APPROVALS

The authors have collected and preserved written ethical approval per international standards.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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